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Detection and Characterization of Lactose-Utilizing *Lactococcus* spp. in Natural Ecosystems

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The presence of lactose-utilizing *Lactococcus* species in nondairy environments was studied by using identification methods based on PCR amplification and (sub)species-specific probes derived from 16S rRNA sequences. Environmental isolates from samples taken on cattle farms and in the waste flow of a cheese production plant were first identified to the genus level, using a *Lactococcus* genus-specific probe. Isolates which showed a positive signal with this probe were further identified to the (sub)species level. *Lactococcus lactis* isolates were also characterized at the phenotypic level for the ability to hydrolyze arginine, to ferment citrate, and to produce proteases and bacteriocins. With specific PCR amplifications, the presence of sequences related to *citP*, coding for citrate permease; *prtP*, coding for protease; and *nisA* or *nisZ*, the structural genes for production of nisin A or nisin Z, respectively, was verified. By these methods, it was possible to isolate lactococci from various environmental sources, such as soil, effluent water, and the skin of cattle. The strains of *L. lactis* isolated differed in a number of properties, such as the ability to hydrolyze arginine or the absence of *citP*-related sequences, from those found in industrial starter cultures. The results indicate that the majority of the industrially produced lactococci do not survive outside the dairy environment, although natural niches are available. However, from those niches strains with the potential to be developed into novel starter cultures may be isolated.

In the dairy industry, large amounts of lactic acid bacteria are involved in the daily manufacturing of fermented milk products such as cheese, butter, and quark. Strains belonging to the species *Lactococcus lactis* are the most important organisms in the manufacture of these products at moderate temperatures. Large-scale industrial processes rely on the use of starter cultures that have been selected for their performance during milk fermentation and product formation (15). As a result, the variability among strains used in industrial dairy fermentations is low (19). However, some traditional dairy products still rely on spontaneous milk fermentations, which result in a large variety of products with different flavors, consistencies, and microbiological quality. There is a great need in the dairy industry for new production strains which result in different product properties. This can be achieved either by genetic modification of known production strains (8) or by isolation of new strains from natural ecological niches. Both for the application of genetically modified starter strains and to allow for an efficient search for strains from natural ecosystems, it is important to know if and where lactococcal strains survive outside the dairy environment. Previous studies have already shown that it is possible to isolate *Lactococcus* spp. from environments other than raw milk. Early investigations identified the cow and the milking equipment as the source of *L. lactis* in raw milk (6; see reference 21 for a review), although other studies could not confirm these results (12, 24). Most of the investigations at that time were rather controversial due to unreliable identification methods. In more recent studies, the isolation of *Lactococcus* spp. from sources other than raw milk has been reported. *L. lactis* subsp. *hordniae* was isolated from the hindguts of wood-eating termites (22, 23), *L. plantarum* was isolated from frozen peas (2), and *L. piscium* was isolated from diseased fish (28). In addition, the detection of *L. lactis* and *L. garvieae* in clinical samples has been reported (5), in-

TABLE 1. PCR primers used

Primer[a]	Target DNA	Base positions (reference)	Sequence	Size of PCR product (bp)
P1 (S)	16S rRNA	41–60 (14)[b]	GCGGCGTGCCTAATACATGC	
P2 (A)	16S rRNA	338–358 (14)[b]	CTGCTGCCTCCCGTAGGAGT	319 (P1 + P2)
P3 (A)	16S rRNA	686–705 (14)[b]	ATCTACGCATTTCACCGCTA	610 (P1 + P3)
P4 (S)	Citrate permease	795–814 (3)	GGAGTTGGTGCTGGTATTGTG	497 (P4 + P5)
P5 (A)	Citrate permease	1273–1292 (3)	CCAACCCTGCTGTAATAGCAG	
P6 (S)	Protease	1198–1217 (7, 27)	CAACACGGCATGCATGTTGC	393 (P6 + P7)
P7 (A)	Protease	1573–1591 (7, 27)	CTGGCGTTCCCACCATTCA	
P8 (A)	Nisin	−99–−78 (16)	CGCGAGCATAATAAACGGCT	319 (P8 + P9)
P9 (S)	Nisin	201–220 (16)	GGATAGTATCCATGTCTGAAC	

[a] S, sense sequence; A, antisense sequence.
[b] *Escherichia coli* numbering is used.

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dicating that these organisms are probably widespread in the environment and not strictly dairy related. In all of these recent studies, the identification of the strains was based on modern methods, including cell wall component determination, DNA-DNA hybridization, and 16S rRNA analysis.

Recently, we have developed reliable methods for the identification and detection of *Lactococcus* spp. based on specific sequences in the variable regions of 16S rRNA (14). We have applied these methods in combination with a genus-specific probe for the reliable and rapid screening of large numbers of isolates from various environmental samples. To assess the dissemination of starter lactococci used in industrial fermentations, we sampled the waste flow of a cheese production plant. Since the effluent of this particular plant is finally discarded by spraying onto a meadow, samples of the soil and grass were taken also. In order to locate natural niches of *Lactococcus* spp., the presence of these bacteria on the udder and skin and in the surroundings of cows was determined since these animals have been implicated as carriers of these organisms (21). To assess a possible relation with the presence of milk, similar sampling was also done on a farm where only bulls were present. Samples from effluent water from the cheese pilot plant as well as those from whey, milk, feed, soil, and grass were collected in sterile containers. Samples from the cow's udder, the skin and saliva from both cows and bulls, and the milking equipment were taken with sterile swabs premoistened with a sterile physiological salt solution. Dilutions of the samples were directly plated on M17 agar (Oxoid, Hampshire, England) containing 0.5% lactose (LM17). For enrichment cultures, 1 ml (or 1 g for the feed, soil, and grass samples) was added to LM17, and the cultures were incubated at 30°C for 24 h. Dilutions of the enrichments were plated on LM17 agar. Single colonies from the plates were grown overnight in a microtiter plate in 250 μl of LM17. The isolates were first identified to the genus level. The DNA of the cultures grown in the microtiter plates was fixed to a nylon membrane filter (Gene Screen Plus; Dupont, Boston, Mass.) by transferring 100 μl of a lysed cell suspension with a dot blot manifold (Schleicher & Schuell, Inc., Keene, N.H.). The filters contained DNA of cell cultures from colonies obtained from the enrichment cultures and the direct platings. From each environmental sample, 48 colonies from the direct plating and 48 colonies from the enrichment culture were selected.

These filters were subsequently hybridized with a genus-specific *Lactococcus* probe, labeled by nick translation (19), consisting of the amplified V1 and V2 regions of lactococcal 16S rRNA. The probe was obtained in a PCR of a mixture of equal amounts of total DNA from *L. lactis*, *L. garvieae*, *L. raffinolactis*, and *L. plantarum*, using primers P1 and P2 (Table 1), and validated with a collection of strains belonging to the genera *Lactococcus* (20 strains), *Lactobacillus* (6 strains), *Streptococcus* (8 strains), and *Leuconostoc* (7 strains). Under stringent washing conditions, no signal was found with the genus-specific *Lactococcus* probe and DNA of strains from the other genera (data not shown). However, when cell lysates of four *Lactococcus* species were transferred to a filter and hybridized with a general 16S rRNA probe and the genus-specific *Lactococcus* probe, significant hybridization signals were obtained in both cases (Fig. 1A and B). *L. plantarum* was not tested in this way since this species cannot use lactose, which was included as the sole sugar to allow for specific enrichment of lactococci. Results of hybridizations with a general 16S rRNA probe and the genus-specific *Lactococcus* probe are shown in Fig. 1C and D. The results of the hybridization with the general 16S rRNA probe reflect the growth and efficiency of the cell lysis procedure (Fig. 1C).

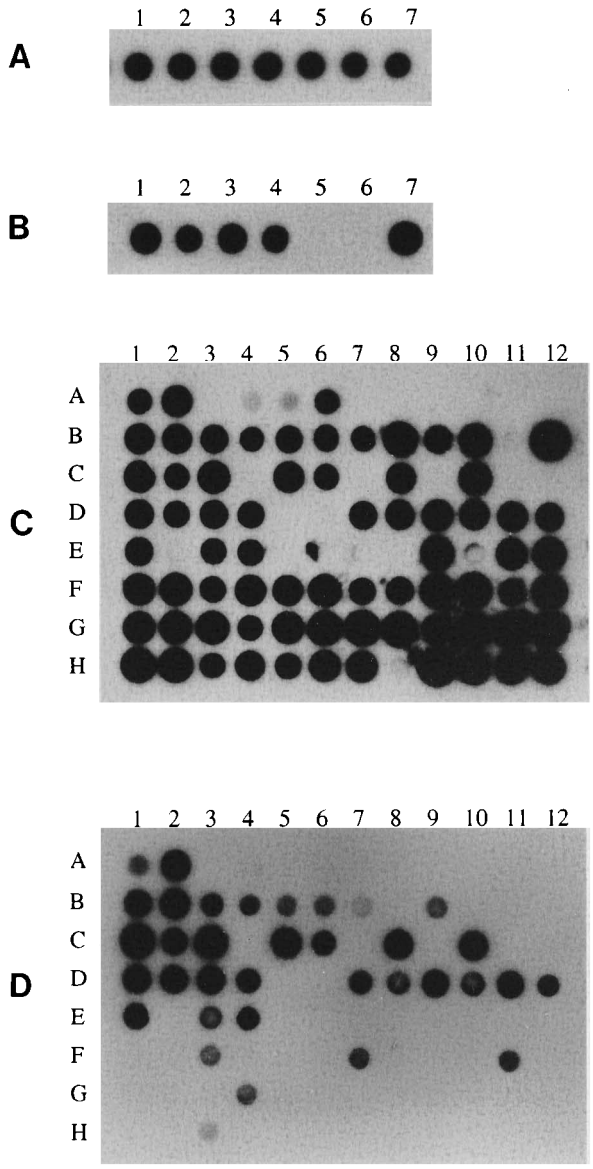

FIG. 1. Validation and use of a *Lactococcus* genus-specific probe based on 16S rRNA. (A and B) Control hybridizations for the four *Lactococcus* strains, *L. lactis* subsp. *lactis* (lane 1), *L. lactis* subsp. *cremoris* (lane 2), *L. raffinolactis* (lane 3), and *L. garvieae* (lane 4), together with *L. casei* ATCC 393 (lane 5) and *L. plantarum* ATCC 245 (lane 6). After lysis of the cultures in a microtiter plate, the DNA was fixed to a nylon membrane. In addition, a mixture of chromosomal DNA, used for generation of the probe, was included as a positive control for the hybridization (lane 7). This membrane was first hybridized with a general probe for 16S rRNA (P1) to check cell lysis and DNA fixation (panel A). Subsequently, the probe was removed and the membrane was hybridized with the *Lactococcus* genus-specific probe (16S rRNA fragment amplified with P1 and P2) (panel B). (C and D) Identification of isolates from a soil sample on the genus level. Lanes A1 and A2 contain DNA from *L. lactis* strains that were included as positive controls. First a hybridization was performed with the general 16S rRNA probe (panel C), and subsequently the blot was hybridized with the *Lactococcus* genus-specific probe (panel D).

Lactococcal isolates were identified with the genus-specific probe (Fig. 1D) and further characterized at the (sub)species level with the (sub)species-specific probes (14). In this way, approximately 80% of the presumed *Lactococcus* isolates obtained from environmental sources could be identified. Isolates that initially showed a signal with two probes, due to impurity

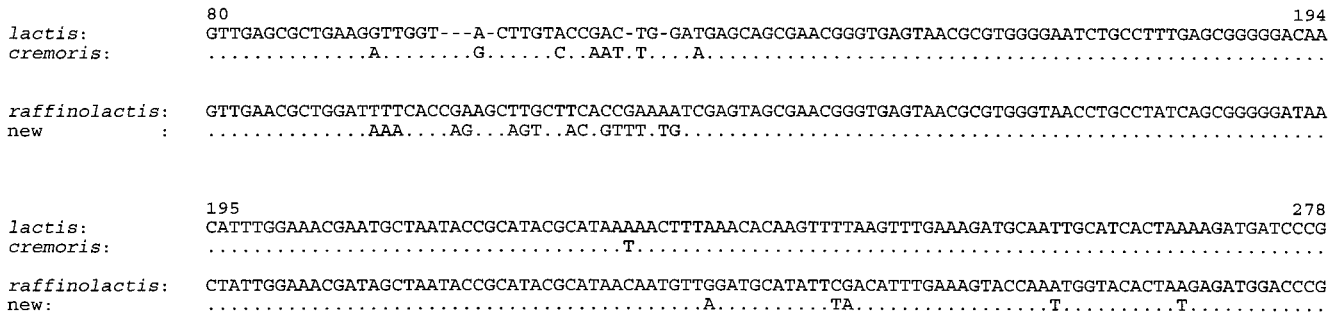

FIG. 2. Alignment of the first part of the 16S rRNA sequence of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. raffinolactis*, and an unidentified *Lactococcus* isolate (new). Only nucleotides which differed with respect to the sequences of *L. lactis* subsp. *lactis* and *L. raffinolactis* are presented.

of the colony, were purified, which always resulted in an unequivocal hybridization signal. The remaining 20% of the isolates were identified by direct sequencing of the first part of the 16S rRNA, using primers P1 and P3 (1). On the basis of this analysis, most of these isolates could be identified as members of the genus *Streptococcus*, in particular, *S. uberis* and *S. parauberis*, which grew to much higher densities than the lactococcal isolates and therefore generated a detectable hybridization signal in spite of the low complementarity of their rRNA to the *Lactococcus* genus-specific probe. Sequence analyses also revealed strains with a 16S rRNA sequence that was very similar to that of *L. raffinolactis* but differed in parts of the V1 region (Fig. 2). These strains probably belong to a new *Lactococcus* (sub)species, and further research is in progress to determine their taxonomic position (13).

In all samples, both the distribution of *Lactococcus* spp. and the estimated total number of lactococci were determined (Table 2). Lactococcal isolates were obtained from all samples, although in many cases only after an enrichment culture, since their initial numbers were low (Table 2), in particular, in the soil and grass samples. In the sampled environments, which are not directly associated with cheese and whey, the *Lactococcus* species *L. lactis*, *L. garvieae*, and *L. raffinolactis* were found to be present (Table 2). There was no difference found in lactococcal content between the samples taken from the cows and those from bulls, indicating that milk is not a prerequisite for the presence of lactococci. Strains belonging to the new (sub) species were isolated also from the wastewater tank and the raw milk, and strains of *S. uberis* and *S. parauberis* were isolated from various samples. The detection of *S. uberis* and *S. parauberis* in most samples derived from the farms is in agreement with the observation that these organisms are frequently associated with cattle and their environment (29).

The cheese milk inoculated with a common mesophilic mixed-strain starter and the whey produced from it contained only arginine-hydrolyzing *L. lactis* subsp. *lactis* and *L. lactis*

TABLE 2. *Lactococcus* species isolated during environmental screening[a]

Sample	No. of isolates[b]	No. of isolates identified as:							Avg concn[c]
		L. lactis subsp. *lactis*	*L. lactis* subsp. *cremoris*[d]		*L. garvieae*	*L. raffinolactis*	New *Lactococcus* sp.[e]	*S. uberis* or *S. parauberis*	
			Arg^+	Arg^-					
Cheese plant samples									
Cheese milk	30[f]	11		24					10^8/ml
Cheese whey	30[f]	30		19					10^6/ml
Waste whey	10 (7)[f]	7 (5)	8 (1)		(1)				10^5/ml
Wastewater tank	24	6	7		4	3	4		10^3/ml
Wastewater disposal site soil	(14)	(5)	(2)			(2)		(5)	$<10^3$/g
Grass	(10)	(4)	(5)		(1)	(1)			
Farm samples									
Raw milk	22	8	2		2	1	7	2	10^4/ml
Milk machine	8 (8)	6 (8)	2						10^4/cm^2
Udder	12	4	6					2	10^3/cm^2
Saliva, cow	(10)	(9)	(1)						
Saliva, bull	(5)	(5)							
Skin, cow	(8)	(4)			(1)			(3)	$<10^2$/cm^2
Skin, bull	(7)	(4)			(3)				
Grass	(14)		(7)			(5)		(2)	
Soil	(30)	(21)			(2)	(1)		(6)	$<10^3$/g
Silage	(10)	(10)							

[a] Values given in parentheses are results obtained from enrichment cultures; other values are from direct plating.
[b] Isolates that gave a positive signal with the *Lactococcus*-specific probe and could be identified with the species-specific probes.
[c] Only results from direct platings are given.
[d] All *L. lactis* subsp. *cremoris* isolates were tested for the ability to hydrolyze arginine.
[e] See Fig. 2.
[f] Total number of isolates is lower than the number of species found because of the presence of double signals.

TABLE 3. Phenotypic and genotypic properties of *L. lactis* subsp. *lactis* isolated from environmental sources

Source	No. of isolates tested	No. with given property[a]					
		Cit$^+$	*citP*	Prt$^+$	*prtP*	Bac$^+$	*nisA* or *nisZ*
Waste disposal land[b]	5	2	0	3	3	0	ND[c]
Soil	5	2	0	0	ND	0	ND
Raw milk	10	0	ND	10	8	1	1
Udder and skin	5	0	ND	5	3	2	2

[a] Cit$^+$, capacity to metabolize citrate; Prt$^+$, capacity to produce proteinase; Bac$^+$, capacity to produce antimicrobial activity.
[b] From soil and grass of the area sprayed with wastewater from the cheese plant.
[c] ND, not done.

subsp. *cremoris*, which did not hydrolyze arginine. However, it appeared that all *L. lactis* subsp. *cremoris* strains isolated from the wastewater and outside the plant were able to hydrolyze arginine (Table 2). It has recently been established that there is a discrepancy between the phenotypic and genotypic identifications of the two *L. lactis* subspecies (10, 18, 19). Strains with an *L. lactis* subsp. *cremoris* genotype can be grouped into those conforming to the known *L. lactis* subsp. *cremoris* phenotype (i.e., no growth at 37°C, 4% NaCl, and inability to hydrolyze arginine) and those with an *L. lactis* subsp. *lactis* phenotype (i.e., growth at 37°C, 4% NaCl, and able to hydrolyze arginine) (10, 18). Therefore, we tested all strains containing the *L. lactis* subsp. *cremoris* genotype for the ability to hydrolyze arginine (Table 2) as an indicator of the *L. lactis* phenotype. The results indicate that strains of *L. lactis* subsp. *cremoris* present in industrial starters and showing the typical *L. lactis* subsp. *cremoris* phenotype are not retrieved from the waste flow of a cheese production plant and other nondairy environments. Probably, the range of organisms in the starter cultures has narrowed due to the selection for better industrial performance, resulting in special varieties of *L. lactis* subsp. *cremoris* which have lost properties that are important for their survival outside a dairy environment. By using an in vitro effluent system, it was shown that *L. lactis* subsp. *cremoris* isolates from starters were outcompeted by environmental isolates (13). This suggests that strains with the typical *L. lactis* subsp. *cremoris* phenotype used in the production of fermented milk products would not be easily isolated from environmental sources, although Salama et al. (19) reported the isolation of strains from supposedly wild fermentations that showed a phenotype closely resembling that of the starter strains.

Some *L. lactis* strains isolated from environmental sources show industrially important properties such as the capacity to metabolize citrate or to produce proteases and bacteriocins (Table 3). A number of *L. lactis* strains isolated during this study were analyzed at both the phenotypic and genotypic levels. The ability to ferment citrate was measured with WACCA medium according to the method of Galesloot et al. (9). The presence of protease activity was determined by using GMA agar plates (11). The ability to produce bacteriocins was determined as described previously (4, 26), using *Micrococcus flavus* and *L. lactis* subsp. *cremoris* SK11 as indicator organisms.

Using specific primers in PCR reactions on the DNA extracted from the isolates, we determined the presence of the *citP* gene, coding for citrate permease (3); the *prtP* gene, coding for the cell envelope proteinase (7, 27); and the *nisA* or *nisZ* gene, the structural genes for production of nisin A or nisin Z, respectively (4, 16) (Table 1). For all primer combinations, a standard PCR protocol was used: 94°C, 1 min, denaturation; 55°C, 1.5 min, annealing; 72°C, 2.5 min, elongation. The results (Table 3) show that strains with these properties can be found in various locations, including soil. Most of the strains which were positive for protease production on GMA agar contained sequences that could be PCR amplified with the specific primers for the *prtP* gene (Table 1) (7). The fact that some strains which were positive on the GMA agar did not contain sequences related to that of the cell envelope proteinase can be explained by either the presence of unrelated proteases or their ability to satisfy their amino acid requirements in another way. It is noticeable that those originating from the udder and skin from cattle and from raw milk show proteolytic activities. In contrast, strains isolated from soil were mostly nonproteolytic, but some of them were able to ferment citrate. The citrate-fermenting strains, however, did not seem to possess sequences related to that of the *citP* gene coding for citrate permease, which is so far the only carrier found in dairy strains that is involved in the translocation of citrate across the cell membrane (3). More detailed studies elucidated that these strains fermented citrate at a much lower rate than the industrially used *L. lactis* subsp. *lactis* var. *diacetylactis* strain (data not shown). These strains may use a different, possibly aspecific, transport system with a lower affinity for citrate.

Three of 25 strains showed antimicrobial activity against the indicator strains tested. All three of them contained DNA that could be PCR amplified with specific primers for the *nisA* or *nisZ* gene (Table 1). The fact that all three producers of antimicrobial activity appeared to be nisin producers conforms to the expectation, since it is the most common antimicrobial peptide found to be produced by *L. lactis* subsp. *lactis* strains (4). All examined strains from environmental sources were found to be resistant to phages present in whey preparations from industrial cheese plants that used the same starter culture as that analyzed in this study (data not shown) (25).

Using a genus-specific probe in combination with direct platings and enrichment cultures, we were able to isolate *Lactococcus* spp. from various environments, indicating that lactococci can survive outside the dairy environment and that some are able to persist in soil and effluent water, on vegetation, and on cattle. The detection of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* strains in nondairy environments supports the hypothesis that *L. lactis* strains present in starter cultures and in spontaneous milk fermentations originate from cattle and their surroundings (20). However, the isolated strains of *L. lactis* differ from those present in starter cultures in a number of properties, such as the ability to hydrolyze arginine and to grow at 37°C and 4% NaCl for *L. lactis* subsp. *cremoris* isolates or the absence of *citP*-related sequences and the resistance to industrially important phages for the *L. lactis* subsp. *lactis* isolates. In addition, the numbers of lactococci found in the nondairy environments were considerably lower than the amounts which are daily released into the environment due to the industrial production of fermented milk products. This also suggests that most starter organisms are not able to persist in nondairy environments, although natural niches are available. The industrially relevant properties found in *L. lactis* strains isolated from nondairy environments and the resistance of these strains to dairy-related phages may allow their use in the development of new fermented milk products, which is currently being evaluated.

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